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TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL

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TRAIL is a member of the tumor necrosis factor (TNF) family of cytokines and induces apoptosis in a wide variety of cells. Based on homology searching of a private database, a receptor for TRAIL (DR4 or TRAIL-R1) was recently identified. Here we report the identification of a distinct receptor for TRAIL, TRAIL-R2, by ligand-based affinity purification and subsequent molecular cloning. TRAIL-R2 was purified independently as the only receptor for TRAIL detectable on the surface of two different human cell lines that undergo apoptosis upon stimulation with TRAIL. TRAIL-R2 contains two extracellular cysteine-rich repeats, typical for TNF receptor (TNFR) family members, and a cytoplasmic death domain. TRAIL binds to recombinant cell-surface-expressed TRAIL-R2, and TRAIL-induced apoptosis is inhibited by a TRAIL-R2–Fc fusion protein. TRAIL-R2 mRNA is widely expressed and the gene encoding TRAIL-R2 is located on human chromosome 8p22-21. Like TRAIL-R1, TRAIL-R2 engages a caspase-dependent apoptotic pathway but, in contrast to TRAIL-R1, TRAIL-R2 mediates apoptosis via the intracellular adaptor molecule FADD/MORT1. The existence of two distinct receptors for the same ligand suggests an unexpected complexity to TRAIL biology, reminiscent of dual receptors for TNF, the canonical member of this family.
Keywords: apoptosis/caspases/FADD/TNF receptor family/TRAIL

Introduction

Tumor necrosis factor (TNF) is the prototypic member of a family of cytokines that serve important functions in the immune system (reviewed by Cosman, 1994). The members of this family interact with a corresponding set of receptors that form the TNF receptor (TNFR) family (reviewed by Smith *et al.*, 1994). Signals induced by these interactions serve such diverse functions as differentiation, proliferation, activation or induction of cell death by apoptosis (reviewed by Cosman, 1994). Apoptosis is the most common form of physiological cell death (Wyllie *et al.*, 1980) and, together with cell proliferation, governs the homeostasis of tissues (reviewed by Thompson, 1995).

A subgroup of the TNFR family has been shown to be critically involved in mediating apoptosis. The members of this subfamily, which includes TNFR-1, CD95 (APO-1/Fas), TRAMP (APO-3, DR3, WSL) (Chinnaiyan *et al.*, 1996; Kitson *et al.*, 1996; Marsters *et al.*, 1996b; Bodmer *et al.*, 1997) and CAR1 (Brojatsch *et al.*, 1996), are characterized by the presence of an 80 amino acid cytoplasmic death domain (DD), which functions to initiate the intracellular apoptotic signaling cascade. Upon ligand-induced cross-linking of these receptors, a death-inducing signaling complex (DISC) is formed at the DD by recruitment of the cytoplasmic DD-containing molecule FADD/MORT1 (Kischkel *et al.*, 1995). In the case of CD95, the interaction between FADD and the DD of the receptor is direct (Chinnaiyan *et al.*, 1995; Kischkel *et al.*, 1995), whereas in the case of TNFR-1 and TRAMP this recruitment is mediated via the DD adaptor molecule TRADD (Hsu *et al.*, 1995; Chinnaiyan *et al.*, 1996; Bodmer *et al.*, 1997). FADD interacts with FLICE/MACH, the pro-form of caspase-8, through its death effector domain (DED) (Boldin *et al.*, 1996; Muzio *et al.*, 1996). The resulting activation of caspase-8 triggers a proteolytic cascade that ultimately leads to apoptosis (Boldin *et al.*, 1996; Muzio *et al.*, 1996).

The two known apoptosis-inducing receptor–ligand systems, TNF and CD95, have been shown to play a major role in many physiological and pathophysiological situations. TNF is produced mainly by activated macrophages, and lymphotoxin α (LTα) by activated T lymphocytes. The role of TNF and LTα in septic shock, autoimmune disorders and graft-versus-host disease is well established (reviewed by Revel and Schattner, 1987; Cerami and Beutler, 1988; Cohen, 1988; Fiers, 1991). TNF has also been shown to be involved in activation-induced cell death (AICD) in peripheral T cells (Zheng *et al.*, 1995; Sytwu *et al.*, 1996), and TNF antagonists can interfere with up-regulated AICD in HIV-positive individuals (Badley *et al.*, 1997).

The CD95 receptor–ligand system is involved in several important physiological and pathophysiological processes (reviewed by Krammer *et al.*, 1994; Nagata, 1997). Under physiological conditions, CD95 is present on many different tissues, while CD95L expression is restricted to activated T cells and sites of immune privilege. CD95L expression by activated T cells leads to AICD (Alderson *et al.*, 1995; Brunner *et al.*, 1995; Dhein *et al.*, 1995; Ju *et al.*, 1995), T-cell cytotoxicity (Kägi *et al.*, 1994; Lowin *et al.*, 1994), virus-induced hepatitis (Galle *et al.*, 1995; Kondo *et al.*, 1997) and auto-immune diabetes (Chervonsky *et al.*, 1997). CD95L expressed at immune-privileged sites, like the anterior chamber of the eye (Griffith *et al.*, 1995), the testis (Bellgrau *et al.*, 1995) or

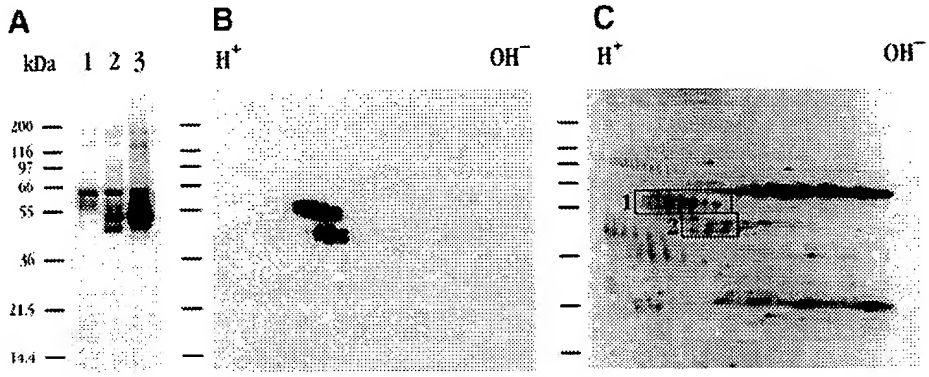


Fig. 1. Purification of TRAIL-R2 from PS-1 cells. Identification of surface proteins binding specifically to TRAIL. PS-1 cells were surface biotinylated, lysed, and proteins were affinity precipitated with anti-Flag M2 (lane 1), Flag-TRAIL or anti-Flag M2 (lane 2 and B) or anti-CD95 (lane 3), and separated by SDS–PAGE (**A**) or subjected to 2D analysis (**B**) before a biotin-specific Western blot was carried out. (**C**) Preparative gel for TRAIL-R2 purification. A cell lysate was prepared from 2×0^{10} PS-1 cells before affinity precipitating with Flag-TRAIL on anti-Flag M2–affigel. Bound proteins were 2D analyzed and silver stained as previously described (Shevchenko *et al.*, 1996). Only the region of the first-dimensional IEF gel strip (pI 4–6.5) containing the spots corresponding to the two identified TRAIL-specific bands (see A, lane 2), as previously determined by 2D analysis (data not shown), was subjected to the second-dimensional SDS–PAGE (B and C). Boxes 1 and 2 in (C) indicate the gel regions cut out for in-gel trypsin digestion and subsequent separate mass spectrometric analysis.

tumors (French *et al.*, 1996; Hahne *et al.*, 1996; Strand *et al.*, 1996), has been demonstrated to mediate immune evasion. Up-regulated CD95 and CD95L expression have also been shown to be causative for increased levels of AICD of T cells from HIV-infected individuals (Badley *et al.*, 1996; Bäumler *et al.*, 1996).

The TNF-related apoptosis-inducing ligand (TRAIL) is the newest member of the TNF family of cytokines (Wiley *et al.*, 1995). Among all family members, TRAIL is most closely related to CD95L (Wiley *et al.*, 1995). Like CD95L, TRAIL induces apoptosis in a wide variety of transformed cell lines (Wiley *et al.*, 1995; Pitti *et al.*, 1996; Mariani *et al.*, 1997) and previously activated T cells (Marsters *et al.*, 1996a). It therefore belongs to a subgroup of apoptosis-inducing members of this family which, in addition to CD95L, also includes the tumor necrosis factors, TNF and LTα (reviewed by Smith *et al.*, 1994). In contrast to the restricted expression patterns of CD95L and TNF, TRAIL mRNA is expressed in a wide variety of normal tissues (Wiley *et al.*, 1995).

In order to better understand the biological role of TRAIL, its apoptosis-inducing potential and the signals triggering the death pathway induced by this ligand, we set out to purify the receptor for TRAIL from cells that undergo TRAIL-induced apoptosis. This led to the identification of a receptor for TRAIL, TRAIL-R2. Recently, a molecule termed DR4 was identified in a private expressed-sequence tag (EST) library based on sequence homology to the DD motif (Pan *et al.*, 1997). Although DR4 protein has not been shown to be expressed by any cell, a soluble Fc fusion protein of its predicted extracellular domain is capable of binding to TRAIL and inhibits TRAIL-induced killing of Jurkat cells (Pan *et al.*, 1997). These results imply that DR4 is a receptor for TRAIL (we therefore refer to it as TRAIL-R1). The results of our studies clearly demonstrate TRAIL-R2 to be distinct from TRAIL-R1 at both the biochemical and functional levels.

Results

Purification of TRAIL-R2

TRAIL-R2 protein was affinity purified from two different cell lines that undergo apoptosis upon incubation with TRAIL, the human T-cell line Jurkat and a spontaneous human B-cell line, termed PS-1. A doublet of biotinylated surface proteins with apparent molecular weights of 52 and 46 kDa, respectively, was affinity precipitated with an N-terminal Flag epitope-tagged version of TRAIL (Flag-TRAIL) (Wiley *et al.*, 1995) from a lysate of surface-biotinylated PS-1 cells (Figure 1A, lane 2) or Jurkat cells (data not shown). Control affinity precipitations with either anti-Flag M2 antibody alone (Figure 1A, lane 1) or the CD95-precipitating monoclonal antibody M3 (Figure 1A, lane 3) (Alderson *et al.*, 1995) did not yield these bands, thus demonstrating the specificity of the respective proteins for TRAIL binding. Biotinylated proteins of the same size were also TRAIL-affinity precipitated from the human B-cell lines Bjab and Ramos, and the human monocytic cell line U937 (data not shown).

Two-dimensional (2D) analysis of the Flag-TRAIL affinity precipitate from biotinylated PS-1 cells (Figure 1B) or Jurkat cells (data not shown) resulted in the detection of the same two surface proteins, appearing as two rows of spots, with isoelectric points (pI) ranging from 4.5 to 5.5 (Figure 1B). Silver staining of 2D gels of preparative Flag-TRAIL affinity precipitates from lysates of 2×10^{10} Jurkat cells (data not shown) and PS-1 cells (Figure 1C) resulted in the detection of two rows of proteins matching the position of the specific biotinylated proteins (Figure 1C, Box 1 and Box 2). The two rows of spots were excised separately, trypsin digested and analyzed by nano electrospray tandem mass spectrometry (Nano-ES MS/MS). Comparison of the two mass spectra indicated that for PS-1 cells (Figure 2A) and Jurkat cells (data not shown) the same protein was contained within the two rows of spots. The data also demonstrate that the lower molecular weight form (Figure 1C, Box 2; Figure

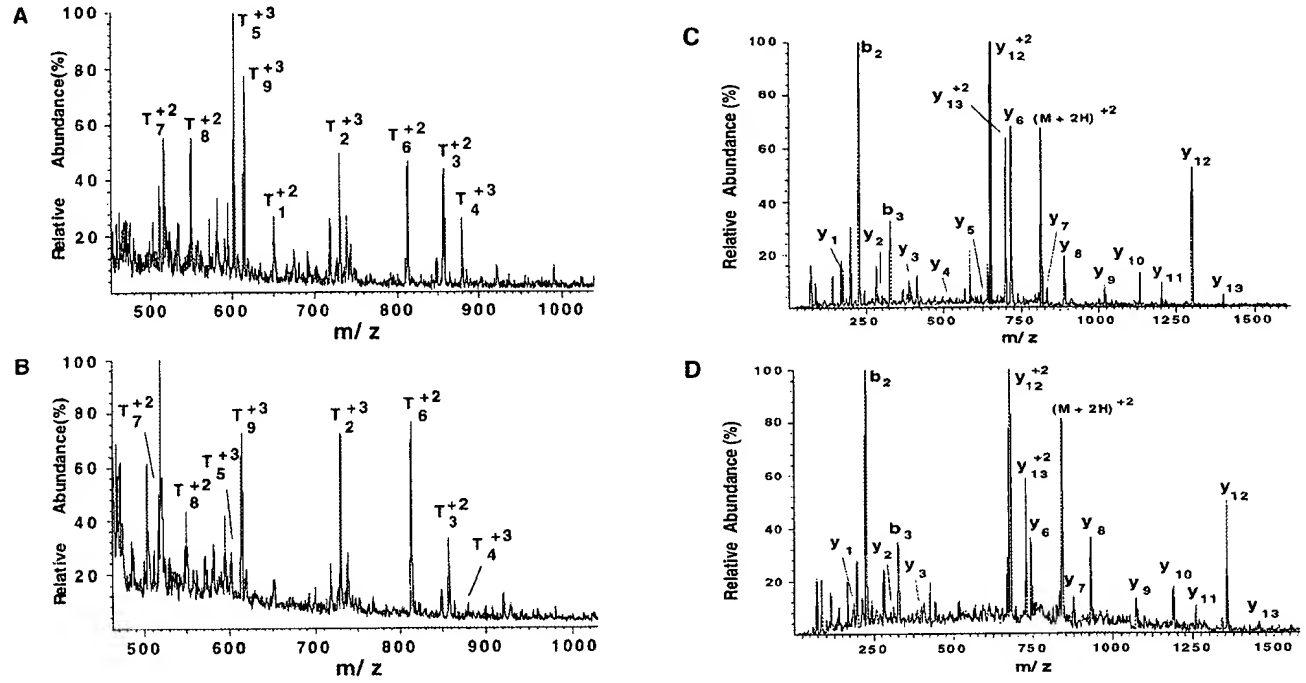


Fig. 2. Mass spectrometric analysis of TRAIL-R2. (**A** and **B**) Mass spectra of peptides released after in-gel trypsin digestion (Shevchenko *et al.*, 1996) of the proteins contained in the gel region indicated in Figure 1C by Box 1 (A) and Box 2 (B). Peptide T1 is present in (A) but not in (B), whereas all other specific peptides (peptides that are not trypsin autocatalysis products) are present in both (A) and (B). (**C**) Tandem mass spectrum of the peptide annotated as T6 in (A) and (B). The Biemann ion nomenclature is used to label the fragment ions that delineate the peptide sequence (Biemann, 1990). The ions labeled '$^{+2}$' indicate doubly-charged fragment ions, and the ion labeled '$(M+2H)^{+2}$' is the precursor ion. (**D**) Tandem mass spectrum of the methyl esterified derivative of peptide T6. Combined analysis of the two tandem mass spectra resulted in sequence determination of this peptide: (L/I)(L/I)VPANEGDPTET(L/I)R (see left column of Table I, for all other sequences determined by Nano-ES MS/MS).

2B) was missing one of the peptides (T1) and thus represents a shorter form of an otherwise identical protein (Figure 2A and B). In addition to determining the mass of nine different peptides, the sequences of six of these peptides were determined either partially (Table I) or entirely (Figure 3A; Table I) by Nano-ES MS/MS. None of these peptide sequences, nor peptide sequence tags, matched any sequence in public databases, indicating they were derived from a heretofore unidentified protein.

Additional peptide sequence information was obtained from a subsequent preparation of this protein from 3×10^{10} PS-1 cells. After trypsin digestion, peptides were separated by reverse-phase high-pressure liquid chromatography (RP-HPLC). N-terminal sequencing of purified peptides and parallel analysis by Nano-ES MS/MS yielded two unambiguous peptide sequences: LLVPANEGDPTETLR and DTLYTMLIK.

Molecular cloning of full-length TRAIL-R2 cDNA

Polymerase chain reactions (PCRs), using combinations of degenerate oligonucleotides derived from these two peptide sequences (arrows in Figure 3A) and PS-1-derived cDNA as a template, yielded a PCR product of 177 bp in length. DNA sequencing of the PCR product and comparison with other members of the TNFR family indicated that it encoded a polypeptide with striking homology to the DDs of TNFR-1, CD95, CAR1 and TRAMP. Screening of a human foreskin fibroblast (HFF) cDNA library with a single-stranded probe of the obtained PCR product resulted in the cloning of the entire coding sequence of human TRAIL-R2 (Figure 3A).

TRAIL-R2 is a novel member of the TNFR family

After isolation of the cDNA for TRAIL-R2, the deduced amino acid sequence was analyzed and compared with other members of the TNFR family. A computer-predicted signal peptide of 55 amino acids is present at the N-terminus of the protein. All non-trypsin-derived peptides (T1–T9; Table I; Figure 3A) detected and sequenced by Nano-ES MS/MS are present in the predicted TRAIL-R2 protein (open boxes in Figure 3A), indicating that the 2D preparations of TRAIL-R2 were pure. Peptide T1 (Table I; Figure 3A), defined and sequenced by Nano-ES MS/MS, is not preceded by a tryptic cleavage site, but instead is located four amino acids C-terminal of the highest scoring computer-predicted signal peptide cleavage site. Thus, the N-terminus of mature TRAIL-R2 is at Ile56. Two cysteine-rich repeats are present in the extracellular domain spanning amino acids 56–210, demonstrating that TRAIL-R2 is a new member of the TNFR family (Figure 3B). No consensus sites for potential N-linked glycosylation are present in the extracellular domain of TRAIL-R2. The cytoplasmic domain (amino acids 232–440) following the transmembrane stretch (211–231) contains a region (345–420) homologous to the DDs of the other apoptosis-inducing members of the TNFR family (Figure 3C). The DD of TRAIL-R2 is 34, 30, 30 and 18% identical to the DDs of CAR1, TRAMP, TNFR-1 and CD95, respectively. Comparison of this new receptor for TRAIL with the recently identified TRAIL-R1 (Pan *et al.*, 1997) revealed that the two proteins are homologous but not identical (58% identity) (Figure 3D). The two receptors for TRAIL are similar in their ligand-binding extracellular

Table I. TRAIL-R2-derived peptide sequences

	Nano-ES MS/MS	Sequence in TRAIL-R2	Observed mass
T1	(L/I)T(Q/K)(Q/K)D(L/I)AP(Q/K)(Q/K)R	ITQQDLAPQQR	1297.2
T2	no sequence information	YGQKYSTHWNDLLFCLR	2188.2
T3	[275]SGEVELS[560]R	CDSGEVELSPCTTTR	1711.4
T4	[215]VC(Q/K)C[1996]R	NTVCQCEEGTFREEDSPEMCR	2634.3
T5	no sequence information	VGDCTPWSDIECVHK	1803.0
T6	(L/I)(L/I)VPANEGDPTET(L/I)R	LLVPANEGDPTETLR	1624.6
T7	(L/I)G(L/I)M[358](L/I)K	LGLMDNEIK	1032.2
T8	[216](L/I)Y[345](L/I)K	DTLYTMLIK	1097.0
T9	no sequence information	DASVHTLLDALETLGER	1839.9

Nano-ES MS/MS-derived sequences and sequence tags are presented in the left column. Identical masses for the amino acids L/I and Q/K, respectively, result in ambiguity of the Nano-ES MS/MS-determined peptide sequence at the positions indicated as (L/I) or (Q/K). Numbers in brackets represent nominal masses of unsequenced amino acids. Peptide sequences as present in the TRAIL-R2 sequence are presented in the center column, and peptide sequences determined unambiguously by combination of N-terminal sequencing and Nano-ES MS/MS are underlined. Observed total masses of the individual peptides are listed in the right column.

domains and also in the portions of their cytoplasmic domains that represent the DD (Figure 3D). In addition, TRAIL-R2 contains sequences adjacent to the transmembrane region that are not present in TRAIL-R1 (Figure 3D). Thus, the data demonstrate that we have identified and cloned a receptor for TRAIL (TRAIL-R2) distinct from TRAIL-R1.

TRAIL binds to recombinant cell surface-expressed TRAIL-R2

To determine whether the cloned cDNA for TRAIL-R2 encodes a functional receptor for TRAIL, we tested whether cells transfected with TRAIL-R2 bind purified, soluble TRAIL. Upon transfection of CV-1/EBNA cells with expression plasmids for either TRAIL-R2 and CrmA or CrmA alone, the TRAIL-R2 co-transfected cells showed binding of TRAIL (Figure 4A). Thus, TRAIL-R2 encodes a functional receptor for TRAIL.

Inhibition of TRAIL-induced apoptosis by TRAIL-R2–Fc and the TRAIL-specific monoclonal antibody M180

Fusion proteins of other TNFR family members have been used successfully in blocking the activities of their respective ligands *in vitro* (Alderson *et al.*, 1995; Brunner *et al.*, 1995; Dhein *et al.*, 1995; Ju *et al.*, 1995) and *in vivo* (Mohler *et al.*, 1993). The molecular cloning of TRAIL-R2 allowed for the production of a protein containing the extracellular domain of TRAIL-R2 fused to the Fc region of human IgG (TRAIL-R2–Fc). In order to test whether the extracellular domain of TRAIL-R2 is sufficient to block TRAIL-induced apoptosis of the human T-cell line Jurkat, we incubated cells with TRAIL either in the absence or presence of TRAIL-R2–Fc, TNFR2–Fc or the mouse monoclonal antibody anti-TRAIL M180, specific for human TRAIL (Figure 4B). Like anti-TRAIL M180, TRAIL-R2–Fc, but not TNFR2–Fc, blocked TRAIL-induced apoptosis in a dose-dependent fashion (Figure 4B). In addition, TRAIL-R2–Fc did not inhibit CD95L-induced apoptosis (Figure 4B). Thus, the extracellular domain of TRAIL-R2 is capable of binding specifically to TRAIL, thereby inhibiting its function.

With the binding of TRAIL to native TRAIL-R2 on Jurkat and PS-1 cells (Figure 1), and to recombinant full-length TRAIL-R2 expressed on transfected cells (Figure 4A), these results demonstrate that TRAIL-R2 is a cellular receptor for TRAIL.

TRAIL-R2 mRNA is widely expressed

To assess in which tissues TRAIL-R2-mediated apoptosis might be of importance, we examined the tissue distribution of TRAIL-R2 mRNA by Northern blot analysis. A single transcript of 4.4 kb was present in all tissues examined, including spleen, thymus, peripheral blood lymphocytes (PBLs), prostate, testis, ovary, uterus and multiple tissues along the gastro-intestinal tract (Figure 5). The cells and tissues with the highest levels of TRAIL-R2 mRNA are PBLs, spleen and ovary, as shown by comparison with control hybridizations with a GAPDH-specific probe.

Chromosomal localization of the human TRAIL-R2 gene to 8p22-21

In order to find out whether any known genetic defect could potentially be caused by an alteration in the gene that encodes TRAIL-R2, we determined the localization of the human TRAIL-R2 gene. PCR screening of two independent radiation hybrid panels mapped the TRAIL-R2 gene to human chromosome 8p22-21, 49 cM from the telomere (data not shown). Sequencing of the PCR product determined that the amplified DNA was specific for TRAIL-R2. None of the other known members of the TNFR family has been mapped to this region of the human genome, although the recently identified osteoprotegerin gene (Simonet *et al.*, 1997) also maps to human chromosome 8, but to a distant position (Simonet *et al.*, 1997). Therefore, these two genes do not form a cluster, as has been shown for other members of the TNFR family. Interestingly, two tumor suppressor genes also map to 8p (Takle and Knowles, 1996; Tanaka *et al.*, 1996).

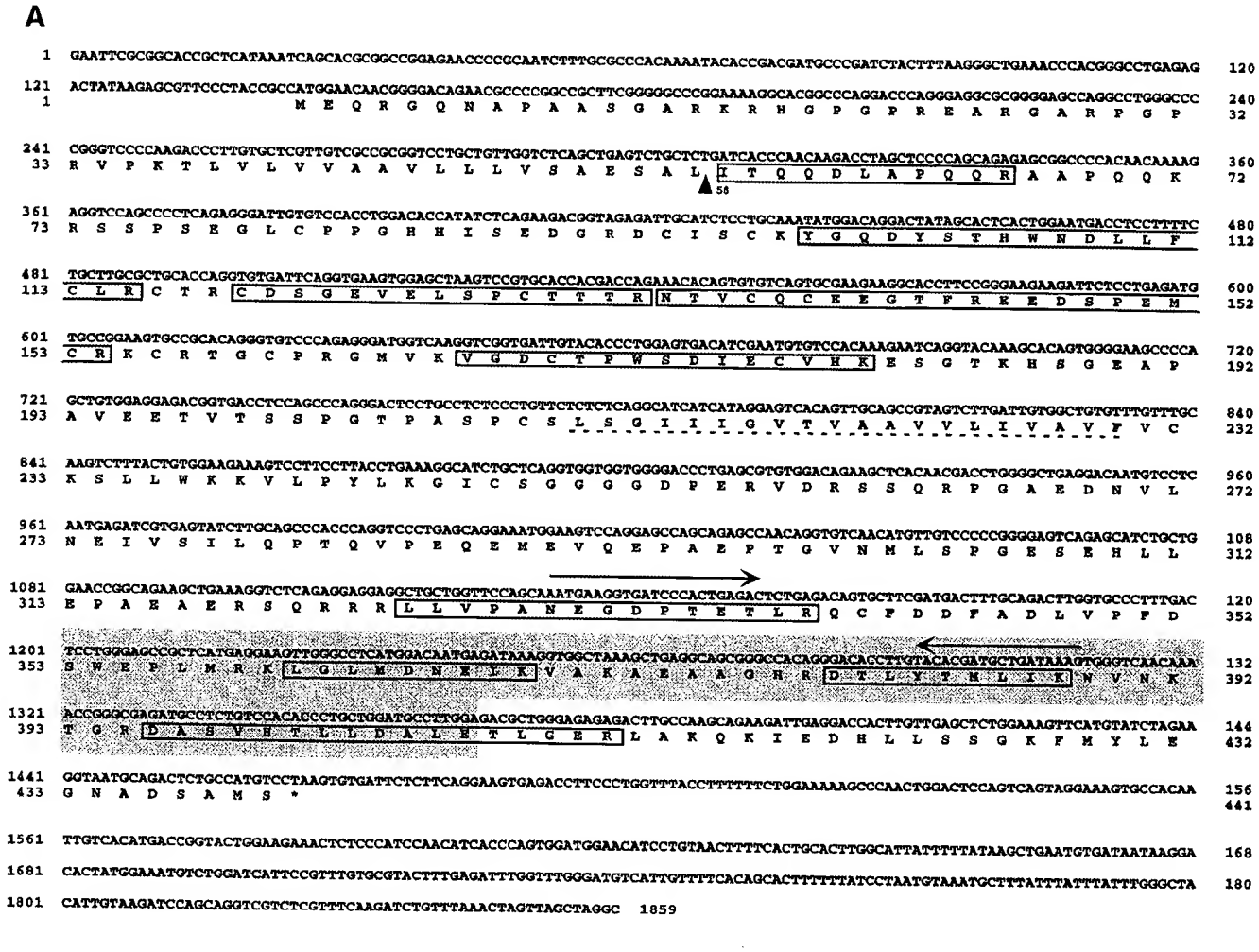
TRAIL-R2 overexpression induces cell death by apoptosis

Other DD-containing members of the TNFR family induce apoptosis upon overexpression in a ligand-independent fashion (Boldin *et al.*, 1996; Chinnaiyan *et al.*, 1996; Marsters *et al.*, 1996b; Muzio *et al.*, 1996; Bodmer *et al.*, 1997). Overexpression of TRAIL-R2 or CD95 resulted in membrane blebbing and nuclear condensation of the transfected CV-1/EBNA cells (Figure 6A). In addition, TRAIL-

R2 overexpression also induced DNA fragmentation (Figure 6B), as determined by measurement of nuclear DNA content and quantification of nuclei with subdiploid DNA content (Nicoletti *et al.*, 1991). Thus, TRAIL-R2 overexpression results in cell death by apoptosis.

TRAIL-R2-induced apoptosis involves caspases and FADD/MORT1

CD95-mediated apoptosis has been shown to involve caspases and the intracellular adaptor molecule FADD/MORT1 (Kischkel *et al.*, 1995; Muzio *et al.*, 1996). We therefore tested whether these molecules might also be involved in TRAIL-R2-mediated apoptosis. Co-transfection of the viral caspase inhibitors poxvirus CrmA or baculovirus P35, or the presence of the caspase-inhibitory peptide zVAD-fmk, was found to inhibit TRAIL-R2-induced apoptosis (Figure 6A and C). This demonstrates that, as with TRAIL-R1, the death pathway engaged by TRAIL-R2 involves caspases. However, in contrast to TRAIL-R1-induced apoptosis (Pan *et al.*, 1997), TRAIL-



R2-induced apoptosis was blocked by co-expression of a dominant-negative form of FADD/MORT1 (FADD-DN) (Figure 6A and C), the intracellular adaptor molecule recruited directly by CD95 and indirectly by TNFR-1 and TRAMP.

Discussion

We have identified a cellular receptor for TRAIL, TRAIL-R2, that is distinct from the recently described TRAIL-R1 (Pan *et al.*, 1997). By virtue of the biochemical cloning approach pursued, we have shown that native TRAIL-R2 is expressed on the surface of both Jurkat cells and PS-1 cells. Recombinant TRAIL-R2 has likewise been shown to be expressed on the surface of transiently transfected CV-1/EBNA cells by binding of TRAIL as determined by FACS staining. Thus, TRAIL-R2 is the first receptor for TRAIL that has been shown to be expressed on the surface of cells in both native and recombinant forms.

TRAIL-R2 and TRAIL-R1 are proteins with similar structures. The N-termini of the two mature TRAIL receptors are at homologous positions since the computer-predicted cleavage site for TRAIL-R1 is after amino acid 51 (amino acid 109 according to Pan *et al.*, 1997), exactly matching the biochemically determined N-terminus of TRAIL-R2 (Figure 3A and D). In this regard, the DNA sequence preceding the codon for Met1 in TRAIL-R1 resembles a well-conserved Kozak consensus sequence, whereas the sequence preceding the previously reported putative start codon for Met58 (Pan *et al.*, 1997) does not (reference: GenBank accession Nos AA102745 and AA100865). The two TRAIL receptors also exhibit homology in their extracellular cysteine-rich repeats, which are important for TRAIL binding, and in their cytoplasmic

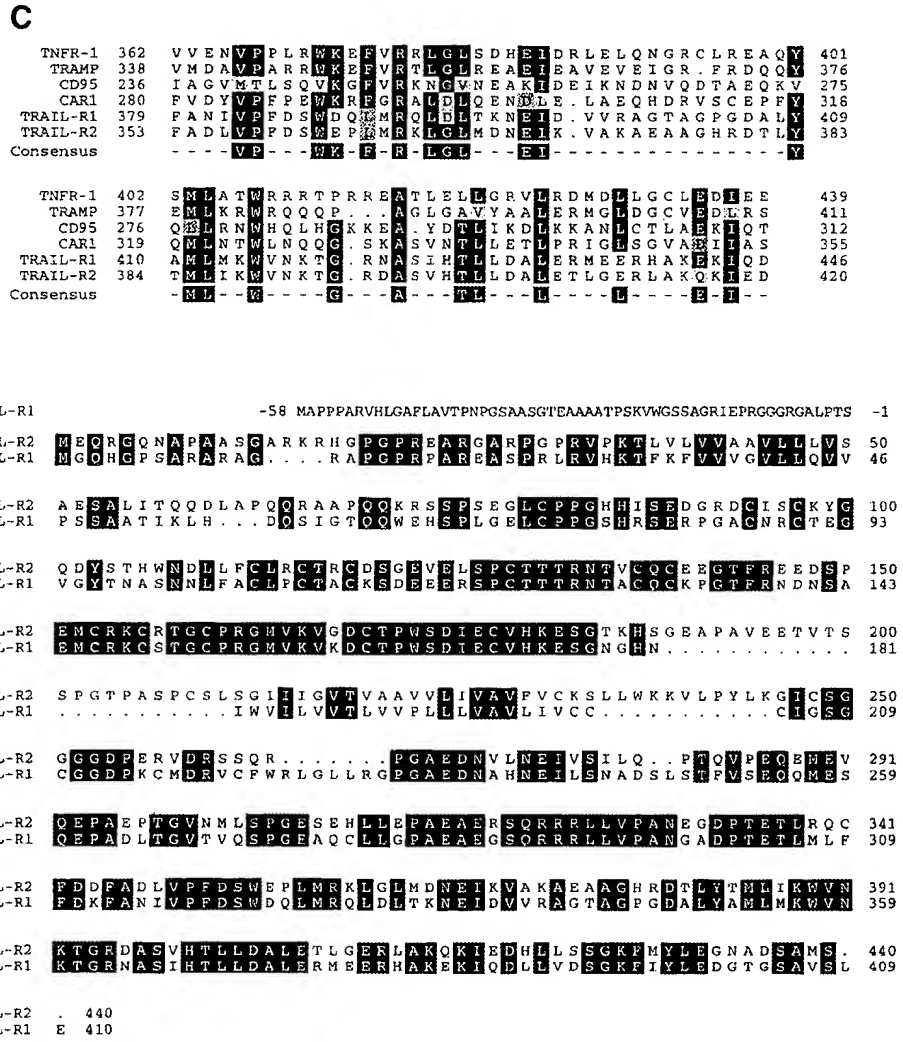


Fig. 3. TRAIL-R2 is a novel member of the TNFR family. (**A**) DNA and amino acid sequence of TRAIL-R2. The N-terminal leader cleavage site is indicated by an arrowhead, the Nano-ES MS/MS-detected peptides by open boxes. The transmembrane domain is marked by a dotted line. The DD is boxed (shaded box). The positions and directions of the two peptide T6- and T8-derived oligonucleotides used for amplification of the TRAIL-R2-specific DNA molecule are indicated by long arrows above the boxed peptides T6 and T8, respectively. (**B**) TRAIL-R2 contains two extracellular cysteine-rich repeats. Extracellular cysteine-rich domain alignment of TRAIL-R2 and other apoptosis-inducing TNFR family members. Conserved cysteine residues are boxed. Three disulfide bonds of linked cysteines found in TNFR1 are indicated above the alignment as SS1, SS2 and SS3. (**C**) TRAIL-R2 has a cytoplasmic DD. DD alignment of TRAIL-R2 and other apoptosis-inducing TNFR family members. Conserved residues are boxed and a consensus sequence is shown. (**D**) Direct comparison of the amino acid sequences of TRAIL-R2 and TRAIL-R1. Identical residues are boxed.

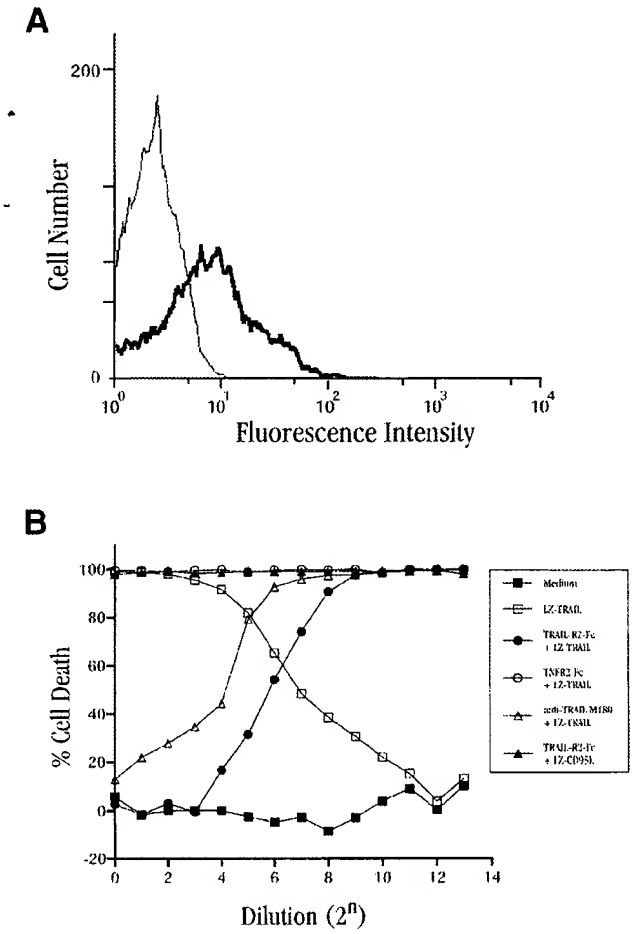


Fig. 4. TRAIL-R2 is a cellular receptor for TRAIL. (**A**) TRAIL binds to cells transfected with TRAIL-R2. CV-1/EBNA cells were transfected with pDC409-CrmA together with pDC409-TRAIL-R2 (thick-lined histogram) or with pDC409-CrmA alone (fine-lined histogram). After 48 h, cells were detached non-enzymatically and stained with LZ-TRAIL, biotinylated anti-LZ antibody M15 and phycoerythrin-conjugated streptavidin. The cells were then analyzed cytometrically. (**B**) Inhibition of TRAIL-induced apoptosis by TRAIL-R2–Fc and a monoclonal antibody to TRAIL. Jurkat cells were treated with serial dilutions of LZ-TRAIL (-□-) starting at 1 μg/ml or were left untreated (-■-). Additionally, LZ-TRAIL was used at a constant concentration of 500 ng/ml (-●-, -○-, -△-) in the presence of serially diluted TRAIL-R2–Fc (-●-), TNFR2–Fc (-○-) or M180 (-△-), starting at 5 μg/ml, and LZ-CD95L was used at 500 ng/ml in the presence of serially diluted TRAIL-R2–Fc (-▲-). Cell death was quantitated as previously described using an MTT assay (Mosmann, 1983).

domains in the region resembling the DD. In contrast to TRAIL-R1, TRAIL-R2 does not contain any potential N-linked glycosylation sites. However, when expressed as soluble Fc fusion proteins, both TRAIL receptors are capable of blocking the apoptosis-inducing activity of TRAIL on Jurkat cells (Figure 4B and Pan *et al.*, 1997). This suggests that the extracellular domains of the two receptors interact with TRAIL in a similar manner. Sequence comparison of the different apoptosis-inducing receptors of the TNFR family shows that TRAIL-R1 and -R2 are most closely related to CAR1 (30 and 37% identity, respectively). Interestingly, besides TRAIL-R1 and -R2, CAR1 is the only other known TNFR family member that contains only two cysteine-rich repeats.

Given that TRAIL is expressed by many different

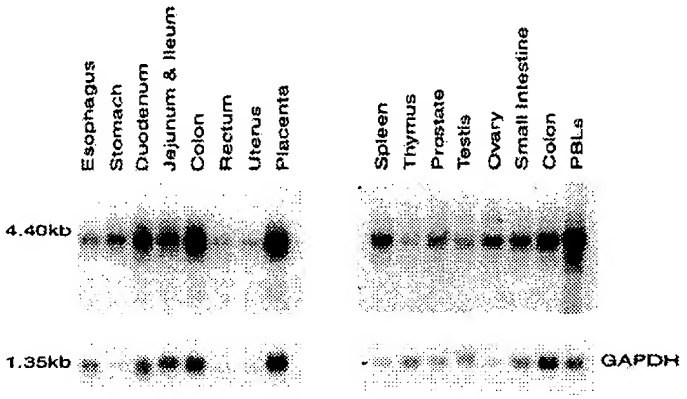
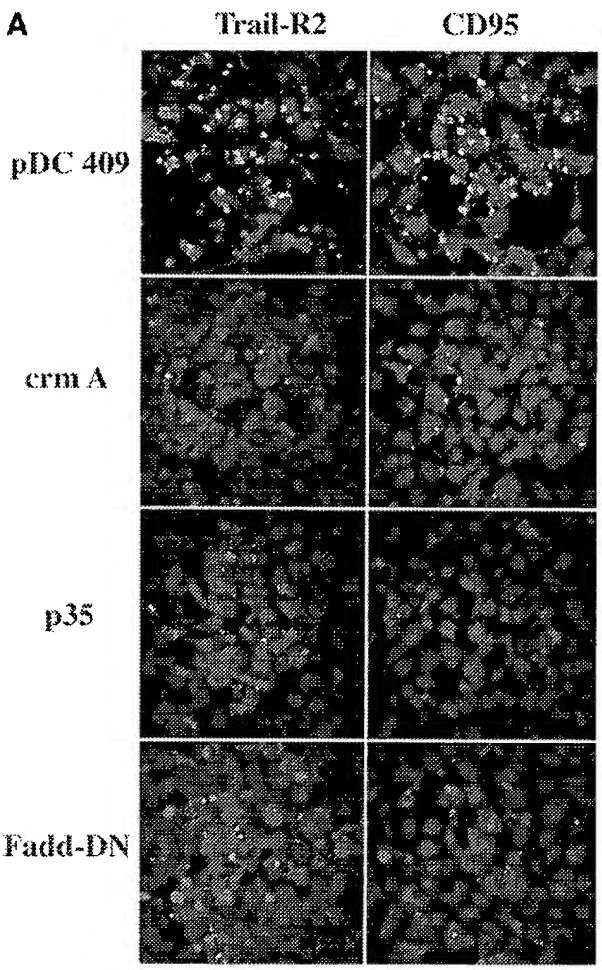


Fig. 5. TRAIL-R2 mRNA is widely expressed. Northern blot analysis of poly(A)$^+$ RNA of various human tissues. A probe specific for TRAIL-R2 was used under high-stringency conditions. A glyceraldehyde-phosphate dehydrogenase (GAPDH)-specific probe was used to standardize for RNA loadings.

tissues (Wiley *et al.*, 1995), the apparent wide tissue distribution of the TRAIL receptors is surprising. The data indicate that, unlike the CD95 system, which is controlled mainly by the tight regulation of CD95L expression, the control point of TRAIL-induced apoptosis does not seem to be located at the transcriptional level. This switch between susceptibility and resistance to TRAIL-induced apoptosis may, therefore, be controlled simply at the level of surface expression of the respective receptors or the ligand itself. Alternatively, it may be relayed from the cell surface to the intracellular signaling adaptors and possible resistance factors, inhibiting their recruitment to the cross-linked receptor. Also, the sequences adjacent to the transmembrane domain present in TRAIL-R2 that are not shared with TRAIL-R1 (Figure 3D) might serve regulatory functions for TRAIL-R2-mediated apoptosis.

The inhibition of TRAIL-R2-mediated apoptosis by FADD-DN indicates that the apoptotic pathway triggered by TRAIL-R2 involves the intracellular adaptor molecule FADD. Therefore, the proximal portions of the death pathways engaged by the two distinct TRAIL receptors seem to differ from each other in that TRAIL-R2-induced apoptosis involves FADD, whereas TRAIL-R1-induced apoptosis does not. However, CD95 is the only receptor that has been shown to bind FADD directly (Kischkel *et al.*, 1995). It is possible that an intracellular adaptor, like TRADD for TNFR-1 and TRAMP, might mediate the binding of FADD to TRAIL-R2. A different, as yet unidentified apoptotic pathway seems to be engaged by TRAIL-R1 (Pan *et al.*, 1997), although the DDs of the two TRAIL receptors are conspicuously similar.

Two studies previously have addressed the relationship of TRAIL-R- and CD95-mediated apoptotic pathways. The first study (Marsters *et al.*, 1996a) showed that HeLa cells transiently transfected with FADD-DN were sensitive to TRAIL-induced apoptosis, indicating that FADD does not seem to play an obligatory role in TRAIL-induced apoptosis. The second study (Mariani *et al.*, 1997) showed that the mouse myeloma cell line Ag8 is sensitive to TRAIL-induced but not to CD95L-mediated apoptosis, although CD95 was detected on the surface of the cells. Both studies taken together indicate that the proximal signaling pathways for TRAIL-R- and CD95-induced



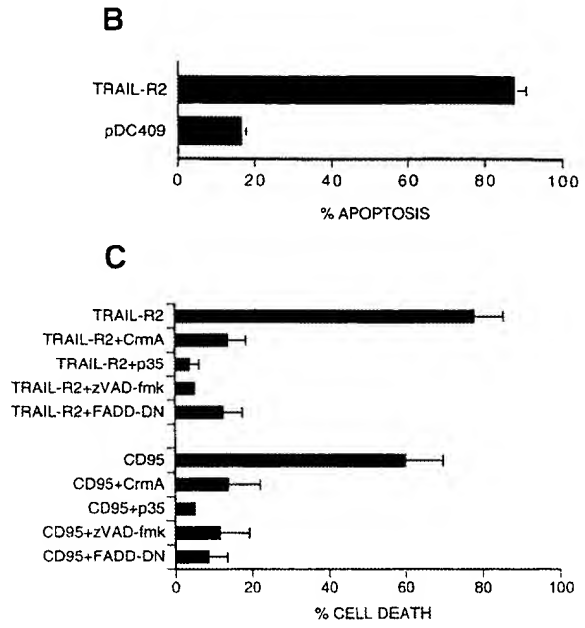


Fig. 6. TRAIL-R2-induced apoptosis is blocked by caspase inhibitors and FADD-DN. (**A**) CV-1/EBNA cells were transfected with expression plasmids for TRAIL-R2 (pDC-409-TRAIL-R2) or CD95 (pDC409-CD95) together with a 3-fold excess of empty expression vector (pDC409) in the presence or absence of z-VAD-fmk (10 μM) or together with a 3-fold excess of pDC409-CrmA, pDC409-p35 or pDC409-FADD-DN. The cells were assayed 48 h after transfection by confocal microscopy as described (Wiley *et al.*, 1995). (**B**) CV-1/EBNA cells transfected with pDC409-TRAIL-R2 or pDC409 were harvested 72 h after transfection. Apoptotic cell death was quantified as described (Nicoletti *et al.*, 1991). (**C**) Four hundred ng/slide of an expression vector for the *E.coli lacZ* gene was co-transfected together with all DNA mixes as in (A) and the cells assayed 48 h after transfection as described (Mosmann, 1983). The values plotted represent the mean and standard deviation of at least three separate experiments.

apoptosis may differ in some cell lines. The development and use of specific antibodies to the different TRAIL receptors will be key to resolving and dissecting the death pathways engaged by the two different receptors.

Failure of homeostasis due to decreased levels of apoptosis can lead to the development of a tumor (Thompson, 1995). The chromosomal localization of the human TRAIL-R2 gene to a region where tumor suppressor genes have been located is very interesting, and future studies will have to address whether one of the two tumor suppressor genes mapped to human chromosome 8p affects the TRAIL-R2 gene.

For many biological processes that involve apoptosis, the effector mechanisms remain unsolved. Among these are such important physiological processes as negative selection in the thymus (reviewed by Sprent *et al.*, 1996), maintenance of tissue homeostasis (reviewed by Thompson, 1995) as well as pathophysiological mechanisms, such as the increased basal levels of apoptosis of non-infected T cells from HIV-infected individuals (Ameisen *et al.*, 1994). The increase in AICD observed in T cells from these individuals is mediated via the CD95 and TNF systems (Bäumler *et al.*, 1996; Badley *et al.*, 1997). However, another death mechanism may be involved in the increased basal levels of spontaneous apoptosis of non-infected T cells from HIV-infected individuals. The TRAIL-specific inhibitors described in this study can now be used to determine whether the TRAIL/TRAIL-R system serves as an effector mechanism in any of these physiological and pathophysiological processes that involve apoptosis.

The identification of a second cellular receptor for TRAIL that induces apoptosis through a pathway different from that of TRAIL-R1 is an intriguing finding. It adds unexpected complexity to the functional role of TRAIL in physiological and pathophysiological situations, reminiscent of dual receptors for TNF and LTα. The existence of a second receptor for TRAIL also raises the question of the rationale for two TRAIL receptors that induce apoptosis. One possible explanation may be that two genes encoding distinct receptors that signal apoptosis through different proximal pathways increase the availability, diversity and versatility of TRAIL-induced apoptosis. Additionally, the presence of both receptors in one cell would allow for simultaneous triggering of two different apoptotic pathways, thereby assuring deletion of an unwanted cell even in the case of a mutation in one of the receptors or downstream signaling effectors. Thus, the TRAIL/TRAIL-R system could be a safeguarded and efficient mechanism for tissue homeostasis. Future studies will also have to address the question of the possible existence of another ligand for these two receptors, as is

the case for the two TNF receptors that bind both TNF and LTα. Together with the reagents presented in this study, antibodies specific for either TRAIL-R1 or TRAIL-R2, as well as mice deficient for either the ligand or receptor(s), will be as important in the elucidation of TRAIL biology and the relative contributions of the two different receptors as they still are in the dissection of the functions of the two TNF receptors.

Materials and methods

Cell culture

The human B-cell line PS-1 is a newly derived, spontaneous B-cell line. All other cell lines referred to herein have been deposited in and described by the American Type Culture Collection (ATCC) (Rockville, MD). Chinese hamster ovary (CHO) cells and CV-1/EBNA cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin, streptomycin and glutamine. All other cells were cultured in RPMI 1640 medium supplemented as above.

Preparation of cell lysates, affinity precipitations and Western blot analysis

Cells were washed once with phosphate-buffered saline (PBS) and lysates were prepared by resuspending the resulting cell pellet in 9 vols of lysis buffer (PBS, 1% Triton X-100 and small peptide inhibitors as described by Peter *et al.*, 1995). After 30 min incubation on ice, the lysates were centrifuged twice at 15 000 *g* (30 min each). The resulting supernatant was pre-cleared for 24 h with 10 μl per ml of lysate of a mix of anti-Flag–affigel beads [10 mg anti-Flag M2 antibody (IBI/Kodak, Rochester, NY) covalently coupled per ml of affigel beads according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA) and protein G–agarose beads (Boehringer Mannheim, Germany)]. Pre-cleared lysates were centrifuged as before and then added to anti-Flag-M2–affigel, Flag-TRAIL pre-coupled to anti-Flag M2–affigel beads or anti-Fas M3 pre-coupled to protein G–agarose (both at 5 mg of protein per 1 ml of beads) at 20 μl of beads per ml of lysate, and incubated with the lysates for 1–3 h (for the preparative lysates 100 μl of beads were added to 200 ml of lysate and incubated for 24 h). The affinity precipitates were then washed twice with lysis buffer, twice with lysis buffer containing 250 mM NaCl, and then again twice with lysis buffer. Affinity precipitates were either subjected to 2D analysis or boiled in a standard reducing sample buffer for 3 min and then subjected to SDS–PAGE on 12% polyacrylamide gels (Novex, San Diego, CA). Cell surface biotinylation, Western blot analysis and detection of biotinylated proteins were performed as described (Peter *et al.*, 1995).

Two-dimensional analysis of affinity precipitates

Two-dimensional analysis of Flag-TRAIL affinity-precipitated proteins was performed by combining a first-dimensional isoelectric focusing (IEF) step and a second-dimensional denaturing SDS–PAGE. Beads containing the affinity-precipitated proteins were dissolved in 100 μl of IEF sample buffer [9 M urea, 65 mM dithiothreitol, 2% Triton X-100, 2% Pharmalyte 3–10 (Pharmacia, Uppsala, Sweden)] and incubated for 1 h at 30°C. The supernatant was loaded onto Pharmacia IEF gel strips (18 cm non-linear immobilized pI gradient; Pharmacia, Uppsala, Sweden). IEF gels were run for 4 h at 500 V and subsequently for 30 h at 3000 V. Before the second-dimensional SDS–PAGE, the gel strip was equilibrated according to the manufacturer's instructions. A 6 cm piece of the gel strip containing the pI range of the specific proteins (as determined by second-dimensional SDS–PAGE of all three 6 cm pieces of the 18 cm gel strip) was placed into a 2D well of a 12% SDS–polyacrylamide gel (Novex, San Diego, CA) and electrophoresed for 2 h at 100 V.

Nano electrospray tandem mass spectrometry (Nano-ES MS/MS)

Peptides released from the gels or contained in RP-HPLC fractions were desalted on disposable microcolumns prepared by using 50 μm RP-Poros R2 beads (Perceptive, Framingham, MA) that had been lodged within the narrow stem of a Eppendorf Geloader pipette tip. Approximately 0.5–1.0 μl of a 20% slurry of the beads in acetonitrile was placed inside a Geloader pipette tip that contained 20 μl of acetonitrile. The beads were allowed to settle towards the bottom of the tip before applying a small amount of pressure using a 1 ml plastic syringe. The reverse phase bed thus produced was then washed with 20 μl of acetonitrile, followed by 20 μl of 0.5% acetic acid. The sample was loaded and washed with 10 μl of 0.5% acetic acid, and eluted directly into the nanospray tip in a volume of 2 μl with 60% methanol containing 1% acetic acid. Mass spectra were obtained using a Finnigan TSQ700 triple quadrupole mass spectrometer with a home-built nanospray source. Nanospray tips were obtained from the Protein Analysis Company (Odense, Denmark), and operated as previously described (Wilm and Mann, 1996). For tandem mass spectrometry, the first quadrupole was operated at a resolution sufficient to pass a 4–5 Da wide window, but the third quadrupole was operated at unit resolution. Collision gas was supplied at a pressure of 4 mTorr. Sequence comparisons with known proteins were made using the computer program PeptideSearch (Mann and Wilm, 1994). Methyl esterification of peptides was performed as previously described (Hunt *et al.*, 1986).

Reverse-phase high-pressure liquid chromatography (RP-HPLC)

Gel-extracted lyophilized peptides in 50 μl of 0.1% trifluoroacetic acid (TFA) were separated by RP-HPLC on a 500 μm i.d.×25 cm capillary column packed with Vydac C-18 material (Vydac, Irvine, CA). Elution under an acetonitrile gradient (10% after 5 min, 85% after 105 min) was monitored at 214 nm, compared with a blank portion of gel subjected to the same trypsin digestion and extraction procedures. Individual peaks were collected in separate fractions during the elution.

N-terminal peptide sequencing

N-terminal sequence analysis of peptides in these fractions was performed on a 494 Procise sequencer (ABI, Foster City, CA) according to the manufacturer's instructions.

Cloning of TRAIL-R2 cDNA

The two unambiguous peptide sequences (T6 and T8) were used to derive degenerate oligonucleotides. For peptide T6, eight 23mer oligonucleotides were synthesized, four for the sense orientation, four for the antisense orientation. For peptide T8, 12 different 18mer oligonucleotides were synthesized for each orientation. The sense oligonucleotides for T6 were combined with the antisense oligonucleotides for T8 in PCR reactions with PS-1-derived cDNA as a template. PS-1 cDNA was prepared by using the Pharmacia first-strand Superscript cDNA synthesis kit according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden). PCR conditions were: one cycle, 4 min at 96°C; 33 cycles, 1 min at 96°C, 1 min at 46°C, 2 min at 72°C; and one cycle, 5 min at 72°C. PCR was carried out on a Robocycler (Stratagene, La Jolla, CA). The oligonucleotide combination that gave the specific PCR product of 177 bp was: T6-sense-4: 5′-AAYGARGG-NGAYCCNACTGARAC-3′ T8-antisense-7: 5′-YTTDATCAGCAT-NGTGTA-3′ (D = A, G or T; N = A, G, C or T; R = A or G; Y = C or T). Underlined nucleotides indicate positions where degeneracy of the individual oligonucleotide was reduced by designing the above-mentioned four (T6) and 12 (T8) different specific oligonucleotides, respectively.

The full-length TRAIL-R2 cDNA was isolated from a HFF cDNA library constructed using random primers and inserted into λgt10 using standard methods (Gubler and Hoffman, 1983). A radiolabeled single-stranded PCR probe specific for human TRAIL-R2 was generated using the TRAIL-R2-specific 177 bp PCR fragment as a template and the T8-antisense-7 oligonucleotide described above as a primer. The library was screened by conventional methods (Sambrook *et al.*, 1989) with the generated PCR probe. Hybridization was overnight at 39°C in 50% formamide as previously described (March *et al.*, 1985), and the filters were washed twice with 2× SSC at 42°C for 30 min. Inserts from the purified, positive clones were isolated, subcloned into the pBluescript SK(+) plasmid (Stratagene, La Jolla, CA) as previously described (Wiley *et al.*, 1995) and sequenced by conventional methods.

Construction of expression vectors

For expression of the full-length human TRAIL-R2 protein, the entire coding region was amplified by PCR from one of the cDNA clones isolated from the HFF cDNA library and cloned into the mammalian expression vector pDC409 (Wiley *et al.*, 1995). A soluble form of TRAIL-R2, which encompassed the extracellular domain of TRAIL-R2 (amino acids 1–208) fused to the Fc region of human IgG1, was constructed in pDC409 as previously described (Smith *et al.*, 1993). The DD of FADD (amino acids 80–206), fused to an N-terminal Flag epitope tag, was constructed in pDC409 to give pDC409-FADD-DN. The LZ-

TRAIL expression plasmid was constructed essentially like the described Flag-TRAIL expression plasmid (Wiley *et al.*, 1995), except that the Flag tag was replaced by a modified leucine zipper that allows for stable trimerization (Fanslow *et al.*, 1994). The same human TRAIL cDNA fragment encoding amino acids 95–281 was used for the generation of LZ-TRAIL-pDC409 as had been used for Flag-TRAIL-pDC409 (Wiley *et al.*, 1995). An expression plasmid for LZ-CD95L was generated by replacing the human TRAIL cDNA fragment encoding amino acids 95–281 in the LZ-TRAIL expression plasmid by a human CD95L cDNA fragment encoding amino acids 117–281 (Takahashi *et al.*, 1994).

FACS staining

For analysis of cell surface expression of TRAIL-R2, transiently transfected CV-1/EBNA cells were detached non-enzymatically 48 h after transfection, then incubated with LZ-TRAIL (5 μg/ml), biotinylated anti-LZ M15 (5 μg/ml) and phycoerythrin-conjugated streptavidin (1:400). Cells were analyzed cytometrically on a FACscan (Beckton Dickinson, San Jose, CA).

Production of the monoclonal antibody anti-TRAIL M180

Balb/c mice were immunized approximately every 3 weeks, twice with CV-1/EBNA cells expressing full-length human TRAIL, then five times with purified Flag-TRAIL in Titermax adjuvant (CytRx, Norcross, GA). Two weeks after the last immunization, mice were boosted intravenously with 5 μg of Flag-TRAIL in PBS. Three days later, the mice were sacrificed and spleen cells were fused to Ag8.653 myeloma cells (ATCC) with 50% polyethylene glycol (PEG) solution (Sigma, St Louis, MO). The fused cells were plated into 96-well plates in HAT selection medium. Hybridoma supernatants were screened for antibody production by an antibody capture assay: 96-well plates were incubated overnight with goat anti-mouse Ig (Zymed) and washed twice with PBS; supernatants were added to the wells, incubated for 1 h at room temperature, then wells were washed four times with PBS. Flag-TRAIL labeled with ^{125}I was then added to each well for 1 h, followed by four washes with PBS. Plates were then placed on film overnight at −70°C. Positive wells were tested against an irrelevant flag-tagged protein as well as in a Jurkat-killing assay, as presented in Figure 4B. Cells from positive wells were then cloned and grown in bulk culture. Anti-TRAIL M180, a monoclonal antibody found specifically to inhibit human TRAIL, was purified on protein A–agarose (BioRad Laboratories, Hercules, CA) and stored at −20°C.

Purification of LZ-TRAIL

CHO cell supernatants containing LZ-TRAIL were diluted 5-fold with a 20 mM Tris buffer at pH 8.5 and passed through Q-Sepharose (Pharmacia LKB, Uppsala, Sweden) at a ratio of 1 ml of supernatant to 0.3 ml (1/0.3) of bead volume. The flow-through was then passed over a Fractogel cation exchange column (E M Separations, Gibbstown, NJ) at a ratio of 1/0.06 ml (v/v). After washing the column with five column volumes of the above buffer, a salt gradient of 0–1.0 M NaCl in 20 mM Tris buffer (pH 8.5) was applied. Fractions that, according to SDS–PAGE and Coomassie staining of the resulting gels, contained LZ-TRAIL were pooled, dialyzed against PBS overnight and stored at −20°C.

Northern blot hybridizations

An aliquot of the same radiolabeled probe used in the library screen was added to two different human multiple-tissue Northern blots (Clontech, Palo Alto, CA; Biochain, Palo Alto, CA). Hybridization was overnight at 63°C in 50% formamide as previously described (March *et al.*, 1985). The blots were washed with 2× SSC, 0.1% SDS at 68°C for 30 min.

Chromosomal localization

Two independent panels of radiation hybrids, the Stanford G3 Radiation Hybrid Panel RH01 and the Genebridge 4 Radiation Hybrid Panel RH02.02 (Research Genetics, Huntsville, AL), were used to determine the chromosomal localization of the human TRAIL-R2 gene. Primers (5′-CCACTTGTTGAGCTCTGGAAAGTTC-3′ and 5′-CCAAATCTC-AAAGTACGCACAAACGG-3′) located near the 3′-end of the TRAIL-R2 open reading frame and in the 3′-untranslated region of the TRAIL-R2 cDNA, respectively, shown to reliably and selectively amplify human genomic DNA specific for TRAIL-R2, were used to screen the two panels. The results were submitted electronically to the appropriate servers for linkage analysis.

Apoptosis assay

As a direct measurement of apoptotic cell death, DNA fragmentation was quantified essentially as described (Nicoletti *et al.*, 1991). Briefly, transfected CV-1/EBNA cells were collected after 72 h by pooling cells contained in the supernatant with the adherent cells after trypsinization. Cells were washed once with PBS and then resuspended in a lysis solution containing 0.1% (v/v) Triton X-100, 0.1% (w/v) sodium citrate and 50 μg/ml propidium iodide. The percentage of apoptotic nuclei was determined by flow cytometry after incubation at 4°C in the dark for at least 24 h.

Cell death assay (X-gal assay)

To quantify cell death, CV-1/EBNA cells were co-transfected by DEAE–dextran with expression constructs for the gene of interest plus the *Escherichia coli lacZ* gene. After 48 h, cells were washed with PBS, fixed with glutaraldehyde and incubated with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Cells expressing β-galactosidase stain blue. A decrease in the percentage of stained cells indicates loss of β-galactosidase expression and correlates with death of cells that express the protein(s) co-transfected with the *lacZ* gene. The values plotted represent the mean and standard deviation of at least three separate experiments.

Accession number

The human TRAIL-R2 cDNA sequence has been submitted to the DDBJ/EMBL/GenBank databases under accession number AF016849.

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